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Comparative analysis of the attachment protein gene (H) of dolphin morbillivirus[1]

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Abstract

DMV, dolphin morbillivirus, a paramyxovirus of uncertain origin recently emerged in Mediterranean dolphins. This study presents the complete nucleotide sequence of the hemagglutinin (H) gene including the gene boundaries. The single open reading frame of the DMV H gene encodes a protein of 604 residues which exhibits overall sequence characteristics similar to the H genes of other morbilliviruses. When compared to its closest homologues, measles virus (MV) and rinderpest virus (RPV), DMV has, respectively, 44 and 46% of amino acid residues in identical positions. The primary sequence of the DMV H protein is markedly less conserved than that of the fusion protein. The comparative data at the genomic level correspond with cross-neutralization studies with different morbilliviruses. Retrospective serogical studies dating back to 1983 indicate DMV-like infections in whales of the eastern Atlantic. The presented data support and extend previous studies suggesting that this novel morbillivirus is one of the phylogenetically oldest morbilliviruses known to circulate today. The relationship of DMV and established morbilliviruses to the newly emerged candidate morbillivirus infecting horse and man is discussed.

Keywords: Dolphin; Cetacean; Morbillivirus; Measles; Distemper; DMV-H

In recent years new morbillivirus diseases have emerged. Thus, dolphin morbillivirus (DMV) has recently been identified as a new contagious pathogen causing a lethal disease among striped dolphins in European waters (Domingo et al., 1990; Van Bressem et al., 1993). A similar virus has also been isolated from harbour porpoises of the North Sea (Kennedy et al., 1988a). Only a few years before the appearance of the dolphin epidemics another previously unknown morbillivirus, phocine distemper virus (PDV), manifested its

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highly pathogenic potential by inducing mass mortality among harbour seals in North-Western Europe (Kennedy et al., 1988b; Osterhaus et al., 1988). PDV was also transmitted to land-living carnivores causing severe disease in farmed mink (Blixenkrone-Møller et al., 1992).

Most recently a candidate morbillivirus infecting horses and man was described (Murray et al., 1995a and Murray et al., 1995b). Its relationship to the established morbilliviruses remains to be clarified.

Until recently the *Morbillivirus* genus of the *Paramyxoviridae* family contained four members: measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV) and peste-des-petits-ruminants virus (PPRV). All of these can cause severe disease in their respective hosts. Knowledge of the natural host range remains crucial in differentiating the closely related members of the genus.

The enveloped morbillivirus particle contains two integral surface glycoproteins, the hemagglutinin (H) and fusion (F) proteins in addition to the matrix (M) protein, that forms the inner layer of the envelope. The helical ribonucleocapsid core contains an ssRNA genome of negative polarity which associates with the nucleocapsid (N) protein, the polymerase or large (L) protein and the phosphoprotein (P) protein.

Morbillivirus isolates from dolphins and porpoises have revealed marked differences in nucleotide and predicted amino acid sequences for the N, M and F genes and part of the P gene in comparison to corresponding sequences from the established members of the genus (Barrett et al., 1993; Blixenkrone-Møller et al., 1994, Bolt and Blixenkrone-Møller, 1994; Bolt et al., 1994; Rima et al., 1995). Compared inter se, the cetacean morbilliviruses isolated so far exhibit a very limited nucleotide sequence variation. Their close relationship also holds at the antigenic level. In previous studies we assessed the antigenic differences between the dolphin and distemper viruses (Blixenkrone-Møller, 1993) using a panel of 75 monoclonal antibodies (Mabs) raised against the structural proteins of CDV and PDV (Örvell et al., 1990; Blixenkrone-Møller et al., 1992). Only minor epitopic differences were revealed when comparing reports by others on virus isolates from dolphins and porpoises examined with a part of the Mab panel used in our studies (McCullough et al., 1991; Osterhaus et al., 1992; Welsh et al., 1992; Visser et al., 1993). The most distinct illustration of the unique antigenic make-up of the cetacean morbilliviruses was assessed in tests with Mabs directed against the H protein. Only two out of 27 Mabs were found to cross-react with the examined virus isolates from dolphins and porpoise (data not shown).

To further investigate the molecular properties of DMV, we have analyzed the attachment protein gene (H) and its boundary with the L gene. The presented relationship between DMV and other morbilliviruses at the genomic level was also reflected in the functional relationship as determined in cross-neutralization experiments. Further, our serological data provide evidence for DMV-like viruses in eastern Atlantic cetaceans.

We have previously constructed a cDNA library based on a morbillivirus isolate from a striped dolphin stranded on the Mediterranean coast of Spain in 1990 (Blixenkrone-Møller, 1993; Blixenkrone-Møller et al., 1994). The DMV library was screened for H gene-specific sequences using a radiolabeled cDNA clone (H10) from the corresponding gene of PDV (Kövamees et al., 1991b) using low stringency conditions: 30% formamide buffer at 37°C (Bolt and Blixenkrone-Møller, 1994).

Two positive clones, 7-E1 and I-A7, later determined to contain the entire coding region and parts of the non-coding regions of the DMV H mRNA, were completely sequenced (Sanger et al., 1977) on both strands using primers synthesized corresponding to the sequences obtained.

The sequences of the H gene boundaries were obtained from polymerase chain reaction (PCR) products using genome-sense RNA as a template for initial reverse transcription. RT-PCR and asymmetric reamplifications for direct sequencing of vRNA were carried out under conditions described previously (Doherty et al., 1989; Bolt et al., 1994).

For amplifying the genomic junction between the H gene and the polymerase (L) gene the primers used were DH10A, which corresponds to antigenome sense nt 1553 to 1569 of the H gene (Fig. 1) and MLA which corresponds to genome sense nt 54 to 69 of the polymerase (L) gene of

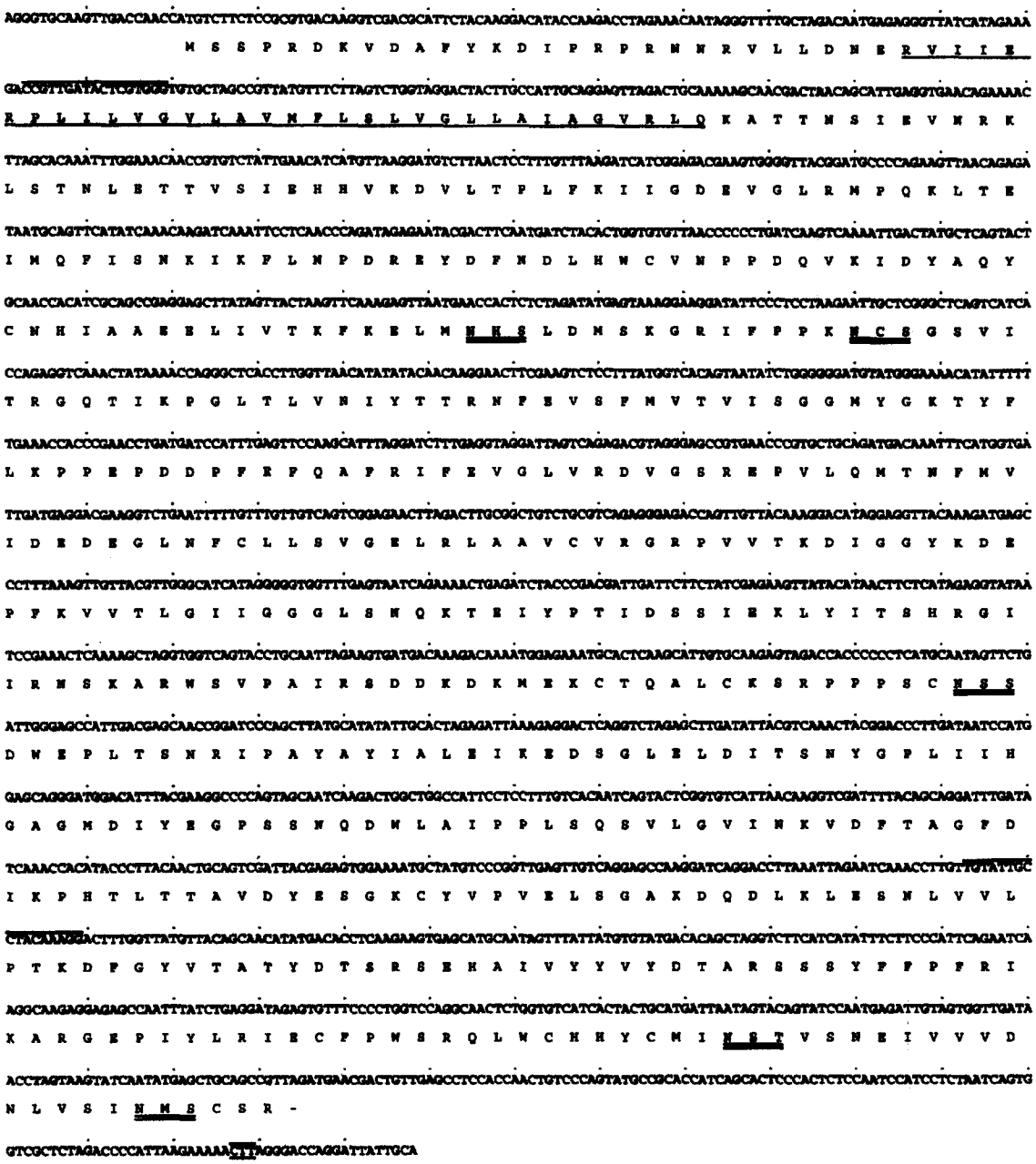

Fig. 1. Nucleotide sequence of the DMV H gene and flanking sequences, including start of the L gene. The positive antigenome sequence is displayed as determined from cDNA clones and PCR products. The intergenic triplet is boxed. The location of the primers in the H gene used to obtain sequences of the gene bounderies are overlined. The corresponding primers selected in the F gene of DMV and in the L gene of MV, respectively, are detailed in the text. Underlining marks hydrophobic region. Potential N-linked glycosylation sites are marked by double underlining.

MV (Blumberg et al., 1988). The location of the primers selected to generate and amplify DNA products of the DMV genome adjoining the 3′ end of the H gene were DF20B, which corresponds to antigenome sense nucleotide 2107-2123 of the F gene of DMV (Bolt et al., 1994) and the genome sense primer, DH20A, located at nt 123 to 139 of the H gene (Fig. 1). All sequences presented were determined for both strands of the original PCR products.

The DMV H gene sequence is shown in Fig. 1, in antigenome (message) sense. The start of the L gene was identified at the 5′ end of the H gene. The compilation of the data from this report with those of our previous studies (Blixenkrone-Møller et al., 1994; Bolt et al., 1994) shows that the physical map of the genome is 3′-N-P/V/C-M-F-H-L-5′ as established for other morbilliviruses (Barrett et al., 1991). The genome of DMV also matches the other members of the genus with respect to the consensus sequences at the gene boundaries (Bolt et al., 1994). The intergenic triplet, CUU (in the positive antigenome sense) is known to be highly conserved for all morbilliviruses, Sendai virus and human parainfluenza virus 3 (Galinsky, 1991; Curran et al., 1992; Blixenkrone-Møller et al., 1994; Bolt et al., 1994; Baron and Barrett, 1995). However, MV and RPV exhibit a CGU triplet at the H-L junction, and for PDV and CDV a CUA sequence has been identified, but interestingly, DMV differs from the other morbilliviruses by having a CUU triplet at the H-L junction (Fig. 1). The intergenic sequences are believed to play a role in the transcription process. Since the L gene of MV is transcribed at a much lower level than the preceeding genes it has been suggested that the triplet at the H-L junction is involved in down-regulation of the L gene transcription (Cattaneo et al., 1987; Curran et al., 1991).

The mRNA transcribed from the H gene is 1946 nt long excluding the poly(A) tail. It contains a single long open reading frame (QRF) covering 1812 nucleotides. The first AUG is located 20 nucleotides from the 5′ end of the mRNA. This start codon is in a moderately favourable context for translational initiation (Kozak, 1986). The corresponding start codons determined for other morbillivirus H proteins are also in suboptimal context for ribosomal binding (Gerald et al., 1986; Yamanaka et al., 1988; Kövamees et al., 1991a; Kövamees et al., 1991b)

The single ORF of the DMV H gene encodes a protein of 604 amino acids with a calculated M_r value of 68 kDa.

The alignment of the DMV H protein sequence with those of other morbilliviruses is given in Fig. 2. The hydropathy profile of the protein shows a considerable similarity to those of other morbilliviruses (data not shown). Thus, the DMV H protein appears to be a class II glycoprotein, where the large hydrophobic membrane anchoring domain of the N terminus is located at exactly the same position as found for other morbillivirus H proteins (Fig. 2). All 13 cysteine residues in the DMV sequence are present in identical positions in RPV and MV, although one (DMV C579) is absent in CDV and PDV (Fig. 2). A total of 17 out of 36 proline residues present in the DMV H protein are found in all other morbilliviruses examined, whereas 75% are conserved in comparison to its closest homologues, RPV and MV. Five potential N-linked glycosylation sites are present in the DMV H protein, two of which are conserved. Thus, the sites at amino acid residue 187 and 391 (Fig. 2) are shared with two (MV, RPV) and three other morbilliviruses (RPV, CDV and PDV), respectively, while the remaining three sites of DMV (residues 172, 582, and 599) are located in close proximity to glycosylation sites found in other morbilliviruses (Fig. 2). Taken together, the glycosylation sites of DMV tend to be evenly distributed throughout the protein, whereas the sites in MV and RPV are in the region close to the membrane anchor and in the distemper viruses they are more C-terminal (Fig. 2).

The amino acid sequence differences between the morbillivirus H proteins are scattered throughout the protein including the cytoplasmic region (Fig. 2). Close to the membrane anchor there is a major area of complete conservation between the H protein of DMV and those of other morbilliviruses (residue 90 to 102, Fig. 2). This area is flanked by regions dominated by conserved residues and conserved substitutions.

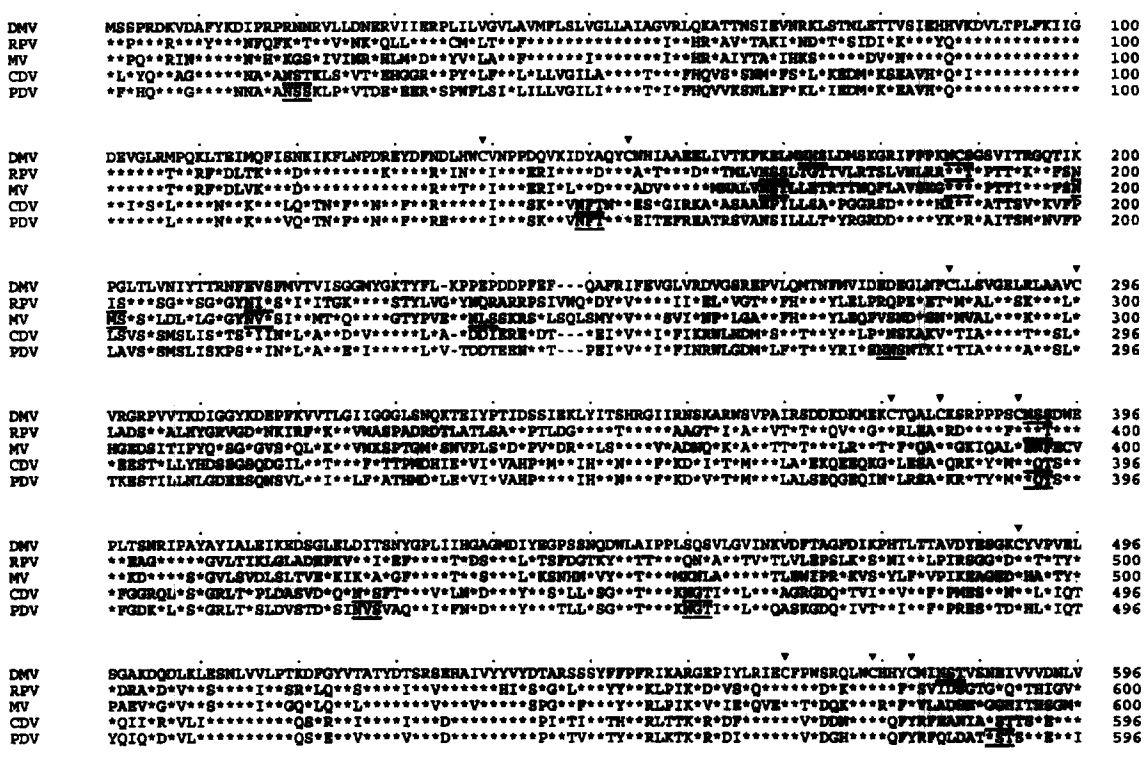

Fig. 2. Alignment of H protein sequences of morbilliviruses. DMV, dolphin morbillivirus; RPV, rinderpest virus; MV, measles virus; CDV, canine distemper virus; PDV, phocine distemper virus. Overlined areas indicate hydrophobic regions; underlined areas represent potential N-linked glycosylation sites; arrowheads mark conserved cysteine residues; asterisks represent amino acid identity. The nucleotide sequences were, when available, obtained from the EMBL database. DMV, this paper; the Convac strain of CDV from Kövamees et al., 1991a (accession no. Z35493); MV consensus sequence was taken from Gerald et al., 1986 (accession no. Z04720); RPV was taken from Yamanaka et al., 1988; PDV from Kövamees et al., 1991b (accession no. Z36979). Multiple alignments were carried out using the CLUSTAL program of the PC GENE package Bairoch, 1992.

The overall amino acid sequence identity between the H proteins of DMV and RPV was 46% compared to 44, 43 and 41% when aligned to MV, CDV and PDV, respectively. Interestingly, the protein sequence identities were less than that at the nucleotide level: the coding region of the DMV H gene displays between 56 and 60% pairwise nucleotide sequence identity when aligned with the corresponding sequences of other morbilliviruses with the highest scores when compared to MV (data not shown).

The H gene product of morbilliviruses is the major antigen inducing neutralizing antibodies. Thus, the cross-neutralizing activity of heterologous polyclonal morbillivirus antisera against cetacean morbilliviruses was examined (Table 1). All antisera exhibited considerable cross-neutralizing titres (80–320) against DMV. The titres were consistently and significantly (at least two dilution steps) lower than those found against homologous viruses. The serological relationships between the morbilliviruses which were isolated prior to 1988 closely support previous studies (Gibbs et al., 1979). Interestingly, the antisera against MV and distemper viruses (CDV and PDV) exhibited significantly higher titers against

DMV than against CDV/PDV and MV, respectively. Thus, at the functional level of neutralization DMV appears to display the closest en bloc relationship to the other morbilliviruses. Taken together, this relationship at the functional level is in line with that found at the genomic level.

Phylogenetic analyses were performed on the nucleotide sequence of the coding region of the DMV H gene and the corresponding sequences of other members of the *Morbillivirus* genus. An unrooted tree (Fig. 3) was constructed with the MEGA PC program (Kumar et al., 1994) using a gamma distance matrix calculated according to the Kimura-2-parameter model (a = 1) (Kimura, 1980) and the Neighbour-joining method (Saitou and Nei, 1987). Alignment gaps were excluded from pairwise distance estimations.

The robustness of the predicted phylogenetic tree was statistically tested using the bootstrap method (Felsenstein, 1985; Hedges, 1992). From the *P* values indicated in Fig. 3, it is apparent that MV/RPV and CDV/PDV form separate clusters. The deduced evolutionary relationships outline

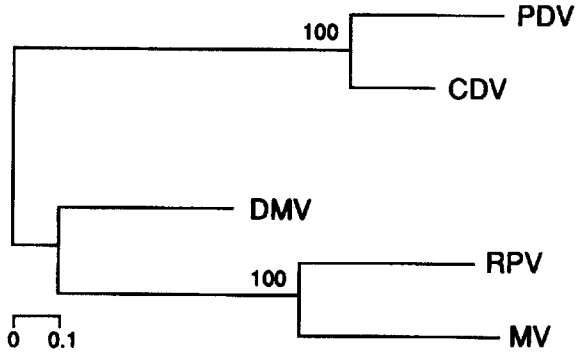

Fig. 3. Phylogenetic analysis of the nucleotide sequences of the coding regions of morbillivirus H proteins. Alignment of the nucleotide sequences was done by use of the PILEUP program of the GCG software package (Devereux et al., 1983; release 8.0) and by hand (data not shown). For details of tree construction refer to text. The branching order represents possible evolutionary relationships between the sequences and the hypothetical common ancestors that existed at the nodes in the tree. Branch lengths are drawn in proportion to the estimated genetic distances as indicated by the bar. The percentages indicate the bootstrap *P* values after 2000 replications (Hedges, 1992). Nucleotide sequences were extracted from the EMBL database (see Fig. 2).

Table 1
The interrelationship of DMV and other morbilliviruses as assessed by cross-neutralization tests[a]

Antiserum[b]	Virus			
	CDV	PDV	MV	DMV
CDV	1280[c]	640	<20	80
PDV	320	>2560	<20	160
MV	<20	80	>2560	320
RPV	40	80	40	80
PPRV	640	320	320	320

[a]The test was carried out as a plaque reduction test in Vero cellcultures in 24-well plates. Serial two-fold dilutions of antiserastarting at 1:20 were tested against 25 PFU of CDV (Onderstepoortstrain), PDV (PDV/DK88), MV (Edmonston strain), and two isolates of DMV, (Dolphin/E90a and b) Blixenkrone-Møller et al., 1994, respectively. All samples were tested in duplicate and conditions were essentially as described by Cremer et al., 1985. The cultures were read on days 5 to 7 post-inoculation after addition of neutral red.
[b]Sera from natural hosts species (i.e. man, cow and goat). Antiserum was kindly provided by I.R. Pedersen (MV) and T. Barrett (RPV, PPRV).
[c]Reciprocal of the lowest serum dilution resulting in a plaque reduction of around 50%.

that DMV is phylogenetically almost equidistinct from established members of the genus. DMV apparently diverged early in morbillivirus evolution. With respect to the estimated genetic distances, DMV seems to be closest to the putative morbillivirus ancestor or archevirus, whereas the other marine morbillivirus, PDV, exhibits the furthest distance. These findings support and extend our earlier studies on the N and F genes (Blixenkrone-Møller et al., 1994; Bolt et al., 1994) and those of others focusing the P gene (Barrett et al., 1993).

Previously, a short nucleotide sequence of the postulated candidate equine morbillivirus (Murray et al., 1995b) displaying limited homologies to M gene sequences of established morbillivirus species was applied to phylogenetic analysis. The authors concluded that the putative equine morbillivirus may represent a very early ancestor in the morbillivirus lineage. Alignment of the reported sequence fragment of the equine virus with M sequences of DMV (Bolt et al., 1994) gives limited homologies (42% and 48% at the nucleotide and amino acid levels, respectively) which

are within the range of those found in similar comparisons with other morbilliviruses (data not shown). When including corresponding sequences of DMV, however, consistent and reliable evolutionary relationships with established morbilliviruses could not be computed (data not shown) indicating that the respective M gene fragment might not be representative enough. Thus, more convincing genetic support for an association of the novel Australian viral pathogen with the morbilliviruses remains to be determined.

To further elucidate the possible origin and spread of DMV, we have tested serum samples from Eastern Atlantic whales for the presence of neutralizing antibodies against DMV. The virus neutralizing assays were carried out as previously described (Blixenkrone-Møller et al., 1991). As shown in Fig. 4, our retrospective serologic investigations provide evidence for DMV-like infections in 13 out of 48 samples from Fin Whales (*Balaenoptera physalus*) more than a decade prior to the discovery of the epidemics among European dolphins.

Recently Duignan et al. (1995a) reported, on serological evidence, that DMV-like viruses are present in various species of dolphins, porpoises and whales from the Western Atlantic and a further report provides serologic evidence for enzootic DMV-like infection among Pilot Whales (*Globicephala* sp.) dating back 13 years (Duignan et al., 1995b).

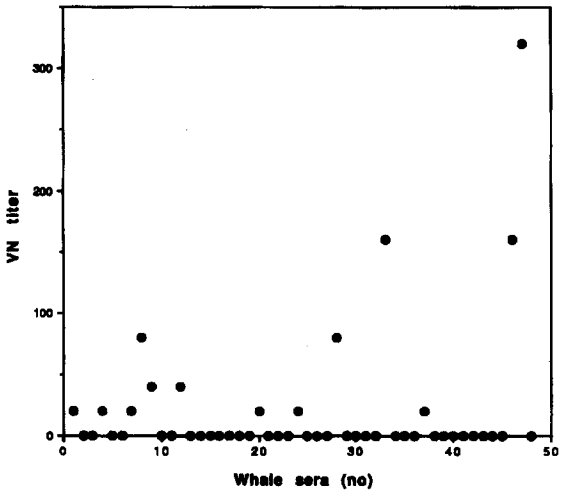

Fig. 4. Serum samples from Fin Whales in neutralization tests against 25 PFU of DMV. The samples were collected in Icelandic waters in 1983. The samples had no detectable neutralizing antibodies against PDV and CDV (data not shown).

It is conceivable that DMV and its ancestors have been circulating among different species of cetacean marine mammals for much more than a decade. However, to the best of our knowledge, it is only in dolphins and porpoises of European waters that the virulence of these cetacean viruses has been recognized (Van Bressem et al., 1993; Visser et al., 1993).

The above findings add to the attractive, but unproven hypothesis that a DMV-like virus has been introduced into the European populations of dolphins and porpoises by migrating species of Atlantic cetaceans. Furthermore, previous findings support the theory that migrating Atlantic harp seals have acted as host and vector for the virus from which PDV has evolved (Goodhart, 1988; Have et al., 1991; Markussen and Have, 1992).

Though the profound genomic distances found between DMV and PDV make a direct epidemiological link between the European morbillivirus epidemics in dolphins and in seals most unlikely, it is possible that the time link between the epidemics is not merely a coincidence. Disturbances of the natural habitat of marine mammals caused by climatic changes, over-fishing and/or environmental pollution are possible common factors that may have led to the close time link between the appearance of the epidemics caused by evolutionary distinct morbilliviruses.

The lack of viral fossils and our limited knowledge of factors of importance for host range and virulence make it difficult to determine the factors influencing the change from a presumed balanced virus-host relationship in infected populations of Atlantic mammals to the devastating epidemics induced by the marine morbillivirus viruses in the European host populations.

In summary, based on the data accumulated in this report and previous papers on the genomic, antigenic and epidemiological levels, we suggest that the isolates from dolphins and porpoises should be recognized as members of a distinct

biological and taxonomic entity: we suggest the species be named cetacean morbillivirus.

The emergence of novel morbilliviruses of uncertain origin further underlines the need to extend our knowledge of the viral and host factors involved in the transmission of morbilliviruses.

The nucleotide sequence reported in this paper have been submitted to the GenBank/EMBL and assigned the accession number Z36978.

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